THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒ Second Renewal ☐ Date: 1/26/73

1. Principal Investigator (give title and degrees):

Richard A. Lerner, M.D., Associate Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Department of Experimental Pathology

4. Short title of study:

Studies on Persistent Viral Infection

5. Proposed renewal date: July 1, 1973

6. How results to date have changed earlier specific research aims:

Some significant observations have been made on continuous lymphocytes established from the New Zealand mice and a unique C-type virus has been isolated. As will be described in detail below, we intend to add to efforts already in progress a number of studies based on this new observation.

7. How results to date have changed earlier working hypothesis:

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Source: https://www.industrydocuments.ucsf.edu/docs/myyh0000

8. Any additional facilities now required? Describe briefly:

As described in detail in the Methods section, we intend to do a large number of studies to determine the number of gene copies of the new virus which we have isolated in mice. Also we intend to determine the evolutionary history of this malignant oncogene. All the studies, and those regarding evolutionary conditions in particular, necessitate extreme precision in determining the melting kinetics of the nucleic acid hybrids. The Acta 5 recording spectrophotometer which we are requesting will enable us to study these parameters. As you will see from the budget, we have not asked for a substantial increase in funds, but rather are asking for this particular piece of equipment and have deleted some other items in order to be able to purchase it. Also, we have now received some funds from the National Science Foundation; however, they are not to be used for the work proposed in this request and at any rate they would not be sufficient to enable us to carry out this problem because of its increased scope. The increase in direct costs is approximately $8,000 to accommodate a two-fold increase in professional staff since our last report.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

As will be described below, our group has increased substantially in size. Two notable additions to the staff are David Kohne and Fred Jensen. We feel that these additions greatly increase our depth in tissue culture and nucleic acid chemistry. The curriculum vitaes of these two investigators are appended.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list.

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/mjyl0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Richard A. Lerner	50%	-0-
Frank J. Dixon, Consultant	--	-0-
David Kohne	100%	-0-
Fred Jensen	100%	-0-
Alvaro Puga	100%	9,527
Stephen J. Kennel	50%	-0-
Bert C. Del Villano	100%	-0-
Technical		
Catherine Morris, Technician	100%	8,618
Part-time services of: secretary, animal caretakers, photographer, glass washers, histology technician, EM technician, machinist & electronic repairman		6,967
	Sub-Total for A	25,112
B. Consumable supplies (by major categories)		
Tissue culture media, glassware and plasticware		6,000
Isotope labeled compounds		3,000
Liquid scintillators and vials		3,000
	Sub-Total for B	12,000
C. Other expenses (itemize)		
Photography, histology, part-time use of electron microscope		4,092
	Sub-Total for C	4,092
	Running Total of A + B + C	41,204
D. Permanent equipment (itemize)		
Beckman Acta 5 spectrophotometer for work in nucleic acid chemistry		17,000
	Sub-Total for D	17,000
E. Indirect costs (15% of A+B+C)	E	6,181
	Total request	64,385

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Source: https://www.industrydocuments.ucsf.edu/docs/myyl0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on persistent viral infection	Council for Tobacco Research #766	$49,495	7/1/72-6/30/73
Molecular structure of the immunoglobulin receptor in continuous cultures of diploid human lymphocytes	National Science Foundation GB 34296	$65,000	6/1/72-11/30/74
Immunopathology of virus infection (Salary - RAL)	NIH Career Development Award AI-46372	approx. $125,000	7/1/70-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Richard A. Lerner, M.D.

Signature Richard A. Lerner Date 1/29/73

Telephone 714/459-2390, ext. 470
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Checks payable to

Scripps Clinic and Research Foundation

Mailing address for check:

Mr. O.K. Kincaid, Controller

Scripps Clinic & Res. Fndn., 476 Prospect St.,
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Responsible officer of institution

Typed Name Edmund L. Keeney, M.D.

Title President and Director

Signature Edmund L. Keeney Date 1/30/73

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Source: https://www.industrydocuments.ucsf.edu/docs/myvl0000

10. Experimental Protocol

A. Introduction

During the first three years of our research program under the auspices of the Council for Tobacco Research-U.S.A., Inc., accomplishments in a number of areas have been made.

First, we decided that since the problems pertinent to the biology of cancer are broad in scope it would be necessary to form a group of investigators with multiple talents to approach the problem. This has now been accomplished and we have a group consisting of individuals with expertise in cell biology, virology, immunology, pathology, medicine, nucleic acid and protein chemistry (curriculum vitaes appended). The collaborative efforts of this group have proven effective since a number of significant gains have been made:

1) A new species of DNA associated with the plasma membrane of lymphocytes has been described and our results have now been confirmed in a number of laboratories.

2) Methods for radioiodination and isolation of polypeptides from the surface of cells have been established. Currently, any plasma membrane associated polypeptide against which one has an antisera can be isolated (see below).

3) 25 different continuously growing lymphocytic cell lines have been established from the NZB, NZW and (NZB x NZW)F_1 hybrid mice. The cell lines were shown to have a marker chromosome and a deletion involving the X chromosome. A new "C type" RNA containing tumor virus was isolated from these cell lines. This virus can be obtained in large amounts, contains reverse transcriptase, and has already been supplied to a number of investigators interested in cancer biology. Of considerable interest is the fact that when 1.0×10^6 syncytial forming units of this virus were injected into newborn (NZB x BALB/c6)F_1 and (BALB/c6 x NZB)F_1 mice they developed both leukemia and lupus erythematosus (see below).

4) Methodology for immunologic study of the fates of nuclear and nucleolar macromolecules has been described. These studies can now be extended (see below).

5) Infection of human lymphoblasts with RD114 virus was accomplished and an interesting biological effect was observed.

In collaboration with Drs. Berge Hampar and Bob McAllister, a series of experiments were undertaken to determine if four different continuous lymphocyte cell lines could be infected with RD114 virus. These experiments were undertaken for two reasons:

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i) It would be technically advantageous if suspension cultures which produce RD114 virus could be obtained

ii) We were interested in the morphological and biochemical effects of this virus on diploid human cells.

Several interesting results were obtained.

First, when the cells and/or culture fluids were assayed by electron microscopy, RNA dependent DNA polymerase, gs antigens, or focus formation, it was clear that they were infected and producing RD114 virus. Thus, the technical objectives of the research protocol were realized. More interesting, however, was the observed alteration in the cell growth pattern and morphology after infection. The cells first formed clumps, then large giant cells with an increase in the ratio of nuclear/cytoplasmic material. This "CPE" in our experience is unique to RD114 virus and has not been seen with VSV, polio, Arbo, Kirsten-Moloney or myxoviruses. The process induced seems to be either syncytial formation and/or endoreduplication of the nucleus but further studies will be necessary to decide between these alternatives. Autoradiographic studies and karyotypes are being done. If it can be confirmed that the process involves endoreduplication of the nucleus then further studies on the molecular mechanisms by which a RNA tumor virus causes a derangement in the control of the "balance" between nucleic acid replication and cytokinesis will be carried out.

An important aspect of these findings is that they suggest, as Hampar has said in his memo of 12/7/72, that "a number of parameters be checked before any attempt is made to inoculate inactivated virus vaccines". This is especially important since exposure of lymphoid cells to RD114 virus presumably inactivated with β-propiolactone leads to rapid appearance of syncytia. Furthermore, after 3 weeks, even using this "inactivated" virus there was evidence that virus replication was occurring.

Our methods for a continuing study of most of the problems outlined above have been detailed in previous reports. In this report we will concentrate on some new approaches which based on our experience of the last three years appear to be technically feasible and of conceptual importance.

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Source: https://www.industrydocuments.ucsf.edu/docs/myy0000

B. Detailed Description and Rationale for Our Additional Research Effort

In addition to continuation of the work that has already been in progress over the last 3 years, we have undertaken a new effort. The conceptual aspects upon which this new approach is based and the methodology with which to study the problem have been developed in our laboratory during the first 3 years of this program.

It is well known that a tumor evokes a cellular and humoral immune response in its host. Until recently it was assumed that the immune response was protective and continued growth of the neoplasm was due to the fact that the immune defenses were overcome by the tumor. However, we now know that antibodies formed in the process of combating the tumor can actually be injurious to the host. The most obvious effect is the direct consequence of synthesis of "enhancing antibodies". These antibodies have been well studied by the Hellstroms and others and are thought to combine with tumor specific transplantation antigens (TSTA) on cell surfaces and in some way interfere with the potentially protective effect of a second cytolytic antibody. A second way in which the immune system may be involved in carcinogenesis is by activation of a latent viral genome. The work of Schwartz, Hirsch and Cornelius has shown that graft vs. host reactions can induce lymphoid tumors in suitable strains of mice. The tumor is of host origin and presumably caused by activation of a latent viral genome because, as Hirsch and Schwartz have shown, the graft vs. host reaction is known to activate an RNA containing tumor virus. A phenomenon which has not been studied as well as those mentioned above is the generation of antibodies and/or phlogogenic antigen-antibody complexes in patients or experimental animals with tumors. These antibodies and/or immune complexes are formed because during carcinogenesis a number of immunogens such as viral polypeptides, nucleic acids, "neo-antigens" on cell surfaces, etc. are generated. A study of the immune response to these immunogens is of extreme importance for two main reasons. First, antibodies and/or antigen-antibody complexes may have an injurious effect on the host. Second, as a research tool antibodies may have predicative value since once the nature of the antigen against which they are directed is known one has some insight into what process the host is attempting to prevent. For example, one of the best places where one might search for candidate human tumor virus is in patients who do not have tumors but in whom there is immunologic evidence that replication of a tumor virus is occurring. Recently, we tested the validity of these concepts in a preliminary study of the New Zealand mice. Since the results of our studies form the basis for this grant request, they are outlined here. The NZB and (NZB x NZW)F_1 mice have a disease which resembles human systemic lupus erythematosus. During the course of their disease these mice make a plethora of antibodies including those to:

dS DNA
SS DNA
dS RNA
SS RNA
nucleolar RNA
DNA-histone complexes
reverse transcriptase
GSA antigen
gs antigen

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In spite of this overwhelming evidence that the host is synthesizing antibody to virtually every structural polypeptide, enzyme, TSTA antigen, or nucleic acid intermediate which an RNA tumor virus might code for or induce during its maturation, these mice seldom get tumors. This, therefore, seemed an ideal model system in which to test our concept that "immunologic tracks" can give clues as to where one can search for oncogenic viruses even and perhaps particularly where the host does not have a tumor. Accordingly, 25 continuous lymphocyte lines were established from NZB, NZW and (NZB x NZW)F_1 mice. Once established, all the lines began to synthesize a virus and had easily demonstrable neo-antigens on their surface. The virus had a C-type morphology, a density of 1.16 gm x cm^{-3}, contained 70S RNA and RNA dependent DNA polymerase and has been designated as the Scripps leukemia virus 60A (SLV 60A). An additional finding of interest was that all cell lines studied had a marker and a deleted X chromosome. Embryos from New Zealand mice were shown to be mosaic for the chromosome marker. It is the purpose of our present effort, for which these funds are requested, to extend these findings in both murine and human systems. Obviously, the interaction between this virus and its host is a complex process and studies ranging from the evolution of this gene in murine genomes to the nature of the immunogens induced by infection are pertinent. Therefore, we have put together a group with expertise in virology, tissue culture, cell biology, immunology, pathology and protein and nucleic acid chemistry to approach the multiple aspects of this problem. The collaboration of this staff is proving effective as evidenced by the progress to date and is the basis of our request for continued support of a broadly based approach to the study of viral oncology with special emphasis on immunoprophylaxis and immunotherapy.

I. The immunopathology of injection of mice with SLV virus

In a preliminary study, (BALB/c x NZB)F_1 and (NZB x BALB/c)F_1 newborn mice were injected with 10^6 syncytial forming units (SFU) of SLV 60A virus. Within 3 months, one half of the mice had developed leukemias and/or lymphomas with generalized involvement of lymphoid tissues and parenchymatous organs. In addition, all animals injected with virus developed strong antinuclear antibodies by two months of age. 3/20 injected mice had glomerular lesions resembling immune complex glomerulonephritis by 3 months.

Thus it appeared that the purified virus preparation might be responsible for both leukemia and autoimmunity. The proposed investigations are designed to further investigate this phenomenon.

(a) Effect of SLV on a variety of murine strains

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Since both the incidence of spontaneous leukemia and autoimmunity are strain dependent in mice, we propose to study a variety of murine strains to determine the incidence of synthesis of anti-DNA antibodies and occurrence of leukemia after injection with SLV virus.

Preparation of virus. Virus will be prepared by isopycnic centrifugation to density of 1.16 gm in the CF-35 zonal rotor. In our hands, approximately 5 liters/hour of supernatant fluid can be cleared in this fashion with a virus yield of approximately 1 mg of virus/liter. Following this, the virus is sedimented to its isopycnic density in a 14.5 to 46.0% sucrose gradient (w/w) at 27,000 rpm for 4.5 hours in the SW27 rotor. Virus prepared in this fashion is then titered for infectivity by syncytial formation in the XC assay and for net yield by determination of the amount of gs-1 antigen present. The XC assay used is that of

Source: https://www.industrydocuments.ucsf.edu/docs/nyy0000

Klement, Rowe, Hartley and Pugh, and the gs-1 protein is determined by a quantitative radioimmune assay developed in this laboratory which is being used routinely and will form the basis for a number of the quantitative studies described below.

(b) Effect of 10^6 synctial forming units of SLV virus in a variety of murine strains

The strains of mice to be studied were selected to include those with both high and low incidences of leukemia and/or autoimmunity. 8 liters of the following murine strains will be used: NZB x BALB/c6, NZB, NZW, (NZB x NZW)F_1, NZW x BALB/c6, C57Bl/6, C57 Brown 10, SWR/J, BALB/c, AJAX. 5 liters from each strain will be injected with 10^6 syncytia forming units of SLV and 3 liters will serve as controls. At 30 day intervals each mouse will be studied for the synthesis of antinuclear antibodies and for proteinuria. When evidence of disease is present the mouse will be bled and a complete autopsy performed. The serum will be studied for the presence of antinuclear antibody and for quantitative determination of the amount of anti dS DNA and anti SS DNA. The methods for determination of antinuclear antibody and quantitation of the amount of anti dS DNA and SS DNA present in the serum are well established and in routine use in this laboratory. White blood cell and red blood cell counts and a differential will be done on all blood samples. In addition to routine sections for pathologic diagnosis, portions of the kidney will be snap frozen for immunofluorescence to determine if IgG, complement, nucleic acids, and gs-1 protein is deposited in the form of immune complexes in the glomeruli. The methodology for a proper immunofluorescent study has been worked out over the last 10 years in this laboratory and adequate facilities as well as appropriate antisera are available. Also, samples of tumor and kidney will be studied by electron microscopy.

(c) Determination of the relationship of the dose of SLV virus to the onset of leukemia and autoimmune disease in the (BALB/c x NZB)F_1 hybrid mouse

Since we already know that when this strain of mice is injected with 10^6 syncytial forming units of SLV virus they develop leukemia and synthesize antinuclear antibody, a more detailed study to determine if a temporal separation in the onset of leukemia and autoimmunity can be achieved.

35 (BALB/c x NZB)F_1 liters will be injected with either 10^6, 10^4, 10^2, 10^1 or 10^0 synctial forming units of SLV virus. Each mouse will be studied as described above.

We expect to learn from the data obtained from these experiments the best combination of viral dose and murine strain necessary to induce both autoimmunity and leukemia. It is further anticipated that by appropriate combinations one may be able to induce autoimmunity without leukemia or vice versa.

II. Relationship of autoimmunity to the amount of gs-1 antigen present in the serum and spleen of ageing New Zealand mice

One of the premises upon which this request is based is that the onset of autoimmunity in the New Zealand mice is related to the replication of RNA tumor virus since during replication this virus presents a genetically susceptible host with a great number of potential immunogenic macromolecules. If we review how the virus replicates, the nature of the immunogenic load

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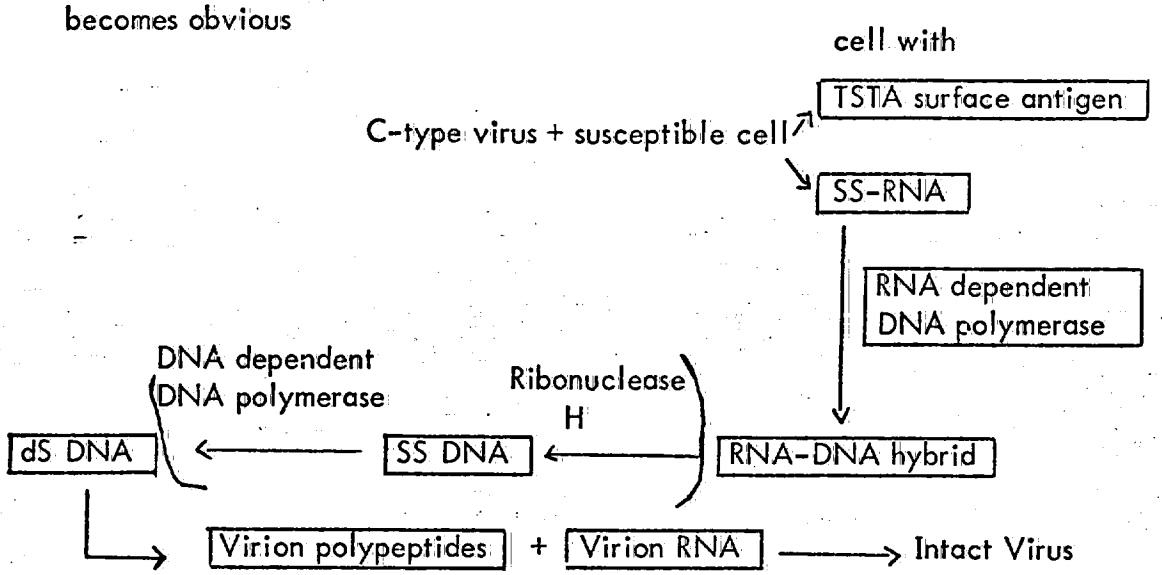
becomes obvious



The immunogens against which the NZB or the (NZB x NZW)F_1 are thought to make antibodies are enclosed in a box [ ] . Of course, in the case of the antibodies against the nucleic acids it is not certain if their foreign nature terminates tolerance to autologous nucleic acids or if they are a specific immunogen.

Regardless, since the autoimmune disease in the (NZB x NZW)F_1 progresses with age, eventually leading to death, it is important to quantitate the amount of virus and/or virion polypeptides synthesized at different ages and correlate this with chemical and pathologic symptoms of autoimmunity.

Methods for determining the amount of antibody against SS DNA or dS DNA in the serum of mice is a technique already worked out and in use in this as well as other laboratories. Also the methodology to make the immunopathologic diagnosis of autoimmunity has been well established by us and other investigators.

Recently we have established methodology for quantitation of the gs-1 antigen in serum and organ extracts. A great deal of effort has been put into development of a radioimmune assay which is sensitive, accurate, highly quantitative, and sufficiently automated so that a large number of samples can be studied. The details of this method are outlined below:

gs Radioimmune Assay

I. Antigens

1. Use purified gs-1. Use IgG from the species in which the anti gs was raised.
2. Iodinate gs with I^{125} to achieve approximately 10,000 cpm per 0.5 ng gs protein (50 µg gs P + 5 mc I^{125} + 5 µg chloramine T -5 min. + 5 µg $Na_2S_2O_5$)
   Iodinate IgG with I^{131} to achieve approximately 10,000 cpm per 50 ng IgG protein. (2 mg IgG P + 4 mc I^{131} + 25 µg chloramine T - 10 min. + 25 µg $Na_2S_2O_5$)

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Post-iodination >95% I* is protein bound (as determined by precipitation with 10% TCA).

3. Prepare stock solutions of I* proteins in BSA (2 mg P/ml in 0.1 M borate buffer, pH 8.3 to 8.5). Stock gs = 1 µg P/ml and stock IgG = 10 µg P/ml. Freeze stock solutions at -20°C in aliquots appropriate for daily use.
4. Prepare working antigen in BSA (2 mg P/ml in 0.1 M borate buffer) so that gs is at 10 ng/ml and IgG is at 1 µg P/ml (1-10 dilutions of stocks). Add 1 µc Na^{22} per ml. 50 µl per test then contains 0.5 ng gs protein and 50 ng IgG protein.

II. Antibody

1. Antibody should be hyperimmune to gs but not necessarily specific for gs, if a pure gs antigen is available for iodination (i.e., antibody to fetal calf serum will not interfere in the quantitation of gs in this assay). We are currently using a guinea pig anti MuLV gs obtained from Dr. Gilden.
2. Dilutions of antibody are made in decomplemented normal rabbit serum, diluted 1-10 in 0.1 M borate buffer.

III. Ammonium Sulfate

Optimum $(NH_4)_2SO_4$ concentration for precipitation of I^{125} gs bound to antibody is dependent on the species in which the hyperimmune anti gs is prepared. With the guinea pig system we are currently using a final concentration of 42.5% ammonium sulfate (4°C) is optimal.

IV. Anti gs dilution for inhibition assay

Since this system is based on the competition of I^{125} gs with cold unknown gs for available antibody sites, it is necessary to carry out the assay in excess I^{125} gs (i.e., in excess antibody small amounts of cold gs would not be defectable). We have chosen as our base line that dilution of antibody which is capable of binding 50% of the I^{125} gs added.

1. Prepare guinea pig anti gs dilutions in 10% ▲NRS, starting with a 1-50 dilution and serial 1-5 dilutions thereafter (i.e., 1-50, 1-250, 1-1250, 1-6250 and 1-31,250)
2. In triplicate add 50 µl diluted antibody plus 50 µl 10% ▲NRS + 50 µl working I* antigen in Beckman microfuge tube (400 µl)
3. Mix and incubate 30 minutes at 37°C and one hour at 4°C
4. Add 100 µl saturated (at room temperature) ammonium sulfate which equals a final concentration of 42.5% ammonium sulfate at 4°C.
5. Mix well and incubate at 4°C for 30 minutes
6. Centrifuge in Beckman microfuge for 5 minutes
7. Aspirate approximately 90% of supernatant, cap and count in Nuclear Chicago automatic gamma counter that is set to record I^{125}, I^{131} and Na^{22} counts.
8. The Na^{22} acts as a volume marker and the I^{131} IgG precipitin acts as a monitor of the technique. The I^{125} specifically bound can be determined. We have programmed a Wang computer to do this calculation directly from the Nuclear Chicago tape read out.
9. On semi-logarithmic paper plot % gs bound on the linear scale versus anti gs dilution on the logarithmic scale. Determine at what antibody dilution 50% of the I^{125} gs is bound.

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V. Control Inhibition Curves

In order to equate the inhibition achieved by unknown materials suspected of containing gs, it is necessary to determine what inhibition is achieved by inhibitors of known concentration. We use a NZB gs obtained from Dr. Gilden that contains 30 µg gs/ml.

Procedure (in triplicate in 400 µl Beckman microfuge tubes)

1. Base line control = no inhibition
   50 µl 10% ▲NRS + 50 µl diluted guinea pig anti gs + 50 µl I* Ag
2. Inhibitors (diluted in 10% ▲NRS)
   50 µl inhibitor of known conc. + 50 µl ab + 50 µl I* ag
3. Mix tubes and incubate at 37°C for 30 min. and at 4°C for one hour
4. Add 100 µl saturated (at room temperature) ammonium sulfate
5. Mix well and incubate at 4°C for 30 min.
6. Centrifuge 5 minutes in Beckman microfuge
7. Aspirate 90% of supernatant, count and determine % I^{125} gs specifically bound
8. Determine % inhibition of each known inhibitor by comparison with base line control

$$\text{i.e., \% inhibition} = \frac{\text{\% bound base line control} - \text{\% bound exp}}{\text{\% bound base line control}} \times 100$$

9. On semi-logarithmic paper plot % inhibition on the linear scale versus added gs inhibitor (ng P/ml). Any inhibition values achieved with unknown substances (i.e., sera, tissue extracts, etc.) can now be quantitated from this standard inhibition curve.

We have already shown that the assay detailed above can be utilized to quantitate the amount of gs-1 antigen in the serum and/or organ extracts of mice. We now propose to bleed NZB, NZW, (NZB x NZW)F_1 serially and determine the amount of gs-1 antigen present in their serum. The same animals will be studied for the development of autoimmune disease. It is anticipated that the amount of gs-1 antigen present in the serum will be a "marker" of the degree of autoimmunity. If this is so, the concept that replication of the virus parallels the development of autoimmune disease will be strengthened.

III. Isolation of virus induced cell surface associated polypeptides from continuously growing New Zealand lymphocytes

One of the antigens against which the NewZealand mice presumably make antibody is a virus induced cell surface polypeptide which in the past has been called the GSA antigen. It is important to know the nature of this antigen because it may be one of the immunogens involved in immune complex formation and might also be a primary target of the host's immune defenses. We were able to show by immunofluorescent studies that it was present on the surface of all the lymphoid lines established from the NZW, NZB, and (NZB x NZW)F_1 hybrid mice. Since these cells can be grown to large numbers and can be harvested as uniform suspensions without the use of proteolytic enzymes, the system is ideal for the isolation of virus cell surface polypeptides. Accordingly, we approached the problem utilizing methodology which has been established over the past two years in this laboratory for radioiodination and isolation of cell surface polypeptides. In a preliminary study, cell surface polypeptides of

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SCRF 60A cells were labeled with ^{125}I by the lactoperoxidase method. Several criteria were considered in setting conditions for effective labeling of these and other cells: 1) constant protein derivatization, 2) maximum iodine incorporation and 3) suitable cell viability. To satisfy the first criterion, we used a constant amount of cold carrier I^- (10^{-5} M). This practice allows a variable incorporation of radioactive iodide (negligible concentration) without changing the number of iodides incorporated per protein molecule. This carrier concentration also allows the enzyme to work in an efficient substrate concentration range. We found that the concentration of H_2O_2 used in the reaction is very important in achieving a large iodide incorporation without oxidizing vital cell components. The optimum concentration varies with the amount of enzyme and substrate in the system. For this reason, standard conditions of 2×10^7 cells/ml, 10^{-5} M KI and 3.3×10^{-7} M lactoperoxidase (Phillips & Morrison, 1970) were adopted. With these set conditions, the effects of H_2O_2 concentration on labeling and cell viability were studied. Maximum incorporation was achieved at 44 µM H_2O_2 with cell viabilities of 90%. This viability increases to 99% when cell washes are done using cold Earle's salt solution rather than room temperature phosphate buffered saline. Multiple additions of this concentration of H_2O_2 did not result in linear increments of I^- incorporation demonstrating that H_2O_2 is not in limiting concentration. Using these conditions, the reaction is over in less than one minute. This rapid labeling allows fast processing and therefore minimal cell exposure to the relatively harsh labeling conditions. Following labeling, the infected cells were disrupted with NP40 and the labeled polypeptides precipitated by the "sandwich" technique. The first antisera was obtained from rats bearing syngeneic Moloney virus induced tumors. Five different batches of this antisera were used. After incubation of the labeled polypeptides with the first antisera for 30 minutes, an appropriate amount of rabbit anti rat IgG was added to precipitate all the rat IgG. Freeze dried immune precipitates were solubilized in 100 µl of 1% SDS containing 8 M urea at 60°C for 1 hour. 1-2% mercaptoethanol was added where reduction of disulfide bonds was required. The sample was layered on the top of 6 x 100 mm 6% SDS polyacrylamide gels and electrophoresis was carried out at 15 ma/gel for 2 hours. Marker proteins labeled with ^{131}I were included in every gel. One mm fractions were sectioned from frozen gels using a Joyce-Lobel automatic gel slicer, counted, and the data corrected for crossover efficiency. Control studies utilizing normal rat sera and a nonspecific immune reaction ("trapping control") were carried out. We were able to reduce nonspecific trapping to about 1% of the labeled protein solution. In addition to radioiodination, cells were labeled with ^3H-glucosamine and processed as described above.

In every experiment, a single polypeptide with a molecular size of approximately 60,000 daltons was isolated. From the experiments with ^3H-glucosamine we were able to show that the polypeptide we have isolated is a glycopeptide. Since this polypeptide only combines with sera from tumor bearing rats and appears only on the surface of cells transformed with RNA tumor virus, it can be classified in the broad group of TSTA antigens. Of course, it is not yet known if this polypeptide is a primary gene product or is coded for by the host genome. If the latter is true or if there is substantial gene related ness among the RNA tumor viruses, this glycopeptide may be a general marker of transformation of cells with RNA tumor viruses.

We propose to study, as described above, the cell surfaces of cells infected with Moloney, Gross, Friend, RD114 and Raüscher virus with a single antisera pool to determine if a similar polypeptide is on the surface of all these cells. From these studies we expect to be able to determine if the polypeptide we have isolated is a general marker of transformation with RNA tumor viruses and if it is present in species other than murine.

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Source: https://www.industrydocuments.ucsf.edu/docs/nyb0000

Also, we would like to know when the New Zealand mice make antibody against this polypeptide. Accordingly, sera from NZB, NZW and (NZB x NZW)F_1 hybrid mice of different ages will be utilized as the first antisera (in the sandwich technique, see above) to isolate the cell surface glycopeptide (TSTA) from continuous lymphocyte lines transformed with SLV 60A virus. In every case the glycopeptide will be studied by gel electrophoresis as described above. From these studies we expect to be able to determine the taxonomic classification of this glycopeptide and how the appearance of antibodies to it in the New Zealand mice correlates with the development of autoimmunity.

IV. Studies on the Structure of SLV Virus

Since NZB and (NZB x NZW)F_1 mice make antibodies to a number of structural virion polypeptides of the murine RNA tumor viruses, it is of considerable importance to characterize the structural polypeptides of SLV virus and determine exactly which of these the New Zealand mice make antibodies against. Two different methods for labeling of the virion polypeptides with isotopes will be utilized. The lactoperoxidase method of iodination modified by attaching the lactoperoxidase to sepharose 4-B beads was found to be effective in labeling intact virions. The large size of the sepharose 4-B beads helps insure that only the polypeptides on the virion surface are iodinated. In addition, all the virion polypeptides will be uniformly labeled with mixed ^{14}C amino acids. Following these two quite different methods of labeling, the virions will be disrupted with SDS. The solubilized proteins will be reduced with mercaptoethanol and characterized by polyacrylamide gel electrophoresis as described above.

In some experiments specific polypeptides labeled by one or the other method will be precipitated by selected antibodies utilizing the sandwich method (see above) (e.g., against gs-1, reverse transcriptase, neutralizing) and then the precipitate will be dissolved and studied by acrylamide gel electrophoresis (see above). From these studies we expect to not only be able to characterize the nature of the virion polypeptides but also, because of the use of specific antibodies, to put individual polypeptides into the proper biological perspective. For example, those polypeptides associated with the virion surface which react with neutralizing antibody are most likely to be important in the host defense against replicating virus.

V. Taxonomy of SLV 60A Virus

It is important to determine the relationship of the SLV virus to other murine RNA tumor viruses. Accordingly, the standard reciprocal neutralization assay will be carried out. The necessary cell lines, viruses and antibodies to carry this out have already been supplied by the NCI.

Of course, because of the "third position effect" in the genetic code, any type of determination of the polypeptide structure does not necessarily confirm a genotypic relationship. Accordingly, the degree of "relatedness" of the SLV 60A genome to other murine genomes will be determined as described below. When these studies are completed, we expect to understand the exact relationship of the SLV 60A virus to other murine RNA containing tumor viruses.

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Source: https://www.industrydocuments.ucsf.edu/docs/myv0000

VI. Evolution of the SLV 60A gene and genotypic relatedness to other murine RNA tumor virus genomes

Since the development of autoimmunity is highly strain specific, it is important to understand how these genes evolved, how many copies are present in the genome of the NZB, (NZB x NZW)F_1 hybrid, and the NZB mice, and the genotypic relatedness of this genome to other RNA tumor viruses. The experimental rationale for these studies is described below.

However, since many of these studies depend on preparing a DNA transcript of the RNA genome of high specific activity to be used as the "probe" to carry out genetic studies, a preliminary study of the feasibility of this using banded SLV 60A virus was carried out.

The virus was found to have a highly active RNA-directed DNA polymerase which readily copies the endogenous template. The rate of incorporation of ^{3}H-TTP is linear for approximately 2 hours. The DNA product has a specific activity of approximately 3.0×10^7 DPM/μg of DNA, and as has been reported, in the presence of actinomycin D, remains associated with 70S RNA. The T_m of the RNA-DNA hybrid product is approximately 77.5°C in 0.14 M $PO_4^=$ as studied by release from hydroxyappetite. This T_m is correct for well matched DNA-RNA hybrids with G+C contents of approximately 40% by the hydroxyappetite method. For example, this is the approximate T_m for ribosomal genes complexed to their RNA gene product when studied by this method. Finally, the recovered product could be shown to reanneal quantitatively with purified 70S viral RNA. Recently it has been reported that the SS DNA transcript is representative of the entire genome and thus a suitable probe for determining gene "copiedness" and genotypic homology among viruses.

Cells cultured from NZB mice produce C-type viruses in large quantity. Many different cell lines from these mice have been established and all of these lines produce C-type viruses in culture. These lines provide a unique opportunity to study many different aspects of the interaction between cell and virus. We plan to study the nucleic acids of these viruses and cells in order to ask some basic questions about the nature of the virus itself, the evolutionary history of these viruses and the virus interaction with cells.

The research proposal here will revolve around 3 separate but related questions: (A) How many different C-type viruses are produced by these cells? (B) What is the evolutionary history of the C-type viruses produced by these cells? (C) How many NZB C-type virus genomes are present in each of the different cell lines and in normal cells from various mouse strains?

Part A may provide us with some idea as to the potential number of mouse C-type viruses which are present in a mouse cell. This has great implications regarding the probability of a cell expressing a virally related tumor or disease. Part B will hopefully provide us with information concerning the origin of viruses. It should also provide a framework which will enable us to judge whether a particular virus is in fact a mouse virus. Part C is important for the interpretation of data obtained in A and B and may also provide us with an indication of the types of molecular interactions which can occur when cells and viruses interact.

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A. How many different C-type viruses are present in these cells? What is the relationship of the NZB virus RNA to other C-type mouse viruses?

It is not as yet clear whether different C-type viruses are being expressed in each of the different NZB cell lines. This question can be approached in at least two different ways, immunologically or by nucleic acid hybridization techniques. In the overall effort of this study both techniques will be tried. The nucleic acid hybridization technique will be more sensitive at detecting differences between the virus. It is possible that two viruses could have exactly the same protein complements and still be different at the level of their nucleic acid sequence. This situation might arise, for example, if at some time during evolution of the mouse several copies of a particular C-type virus genome were integrated into the mouse DNA. Thus, each mouse cell would have several copies of the viral genome. Section C will discuss the determination of the "copiedness" of a viral genome in a cell. Each of these multiple genomes could then diverge from one another with time and at some later time the genomes would be similar but not identical to one another. With enough time and change, a new virus might be formed. The repeated DNA sequence present in all mammals goes through an evolutionary history similar to this.

Two viruses can have exactly the same proteins and different nucleic acid sequences because of a characteristic of the genetic code called synonomous codons. It is known that a sequence of 3 nucleotides codes for an amino acid. The rules of the genetic code are flexible enough so that many amino acids can be coded for by more than one triplet sequence of nucleotides. For example, the amino acid leucine is coded for by the triplets, CUU, CUC, CUA, CUE. It is clear that a mutation could occur in the third position of any one of these four triplets with no change in the amino acid sequence of the protein specified by the gene in which the triplet occurs. Thus, the nucleic acid sequence can change with no concomitant change in the protein. Theoretically, the nucleic acid sequence can change by 18% while no change is seen in the amino acid sequence of the protein coded for. Nucleic acid hybridization techniques will easily detect a 1% difference between two nucleic acid sequences. The rationale for doing this will be explained below.

In order to compare the RNA sequences of different viruses, nucleic acid hybridization techniques must be used. The basic rationale for the hybridization (or reassociation) reaction is seen in Figure 1 and involves the ability of complementary nucleic acid sequences to recognize each other and interact together. This recognition and interaction leads to the formation of a stable double stranded nucleic acid molecule from two complementary single stranded nucleic acid molecules.

Two virus RNA's can thus be compared by mixing the single stranded RNA of one virus with the radioactive complementary strand from another virus and determining whether a double stranded "hybrid" double stranded molecule can be formed. A hybrid double stranded molecule is one in which one strand is form one virus and the other from a different virus. The radioactive complementary strand to be used for these experiments will consist of a radioactive DNA molecule made from the viral RNA by an enzyme, reverse transcriptase, which uses

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an RNA template to make DNA copies of that template. Recent advances in this area make it possible to obtain a full DNA copy of the RNA molecule. The techniques for forming and assaying for double stranded nucleic acids, DNA:RNA or DNA:DNA are well worked out.

One measure of the degree of relatedness between two viruses is the amount of radioactive DNA (which represents one virus) which will form stable double stranded hybrids with the RNA of the other virus. By proper planning, this parameter can reveal the relative size of the two viral nucleic acids being compared as well as some details of the evolutionary events which have formed the present day virus. It can tell, for example, whether new genetic material has been added or deleted from two related but not identical viruses.

A second measure of the degree of relatedness between two viral nucleic acids depends on the fact that single stranded molecules which are only partially complementary can also interact to form a stable double stranded hybrid molecule as is described in Figures 2 and 3. In this case some of the nucleotides in one strand are paired with a non-complementary (see Figure 3 II) nucleotide in the other strand. The degree of similarity of the two complementary strands of a hybrid molecule can be determined by measuring the thermal stability of the hybrid and comparing it to the thermal stability of a perfectly complementary double stranded molecule (see Figures 2 and 3). Thus, a second measure of the degree of relatedness between two viral RNA's is the thermal stability of the hybrid molecule. As is seen in Figures 2 and 3, the quantitative difference between the complementary sequences of the hybrid molecule can then be determined.

Two parameters, then, define the degree of relatedness between two nucleic acids -- (1) the fraction of the nucleic acid from one virus which will form a hybrid with the RNA of another virus and (2) the thermal stability of the hybrid molecule formed. Different combinations of these two parameters can reveal important events which have occurred during the formation of the present day viruses.

The relationship of the NZB virus to other mouse C-type viruses will also be examined as part of this study.

B. What is the evolutionary history of C-type virus nucleic acid with regard to virus-like DNA present in mouse cell DNA?

The practical reason for doing this study is that it might well provide a framework for determining whether the NZB C-type virus is in fact a "mouse" virus. This type of study has evident importance in these days of hunting for a "human" cancer virus among the C-types.

C-type viruses have been isolated from spontaneously transformed cells of many different species of mammals. In all cases examined thus far, the RNA sequence isolated from a particular virus originating from a particular species are also represented in the DNA

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of the species from which it was isolated. DNA sequences homologous to the viral RNA are also found in non-transformed cell DNA of the same species. It is clear that in at least some of these cases the whole viral genome is present in the normal non-transformed cell since it has been possible to activate "normal" cells and cause them to produce C-type viruses characteristic of that species. These data suggest that the virus nucleic acids are transferred in an inactive form from generation to generation, probably in the germ line. This raises the possibility that these viruses have been present in the mouse during a large part of the "mouse" evolution. We wish to study the evolution of the viral like sequences present in the mouse cells, concentrating on the NZB virus nucleic acid. However, other types of C-type mouse viruses will also be examined.

The methods for doing nucleic acid sequence evolutionary studies are well worked out. The basic technique will involve reacting a very small amount of radioactive viral nucleic acid (the same radioactive DNA used in section A) with a large amount of non-radioactive DNA from a variety of species in order to form hybrid double stranded molecules. In this case the hybrid will be composed of one nucleic acid strand from the virus and one strand from the DNA of a mammal. The non-radioactive DNA will be isolated from a variety of mammalian species which have varying degrees of evolutionary relationship to the mouse. Non laboratory strains of these species will be used where possible. The two parameters of relatedness, (1) the fraction of radioactive viral DNA which is capable of forming hybrids with the non-radioactive mammalian DNA, and (2) the thermal stability of the hybrid molecule, will be measured.

The results of these studies should throw light on the origin of viruses. Further, they may provide us with a framework for judging whether a particular present day virus has been associated with a particular animal species during the evolution of the animal species.

C. How many integrated DNA copies of the NZB C-type virus genome are there per cell in the different NZB cell lines and in different mouse strains?

The results of this study are essential for the proper interpretation of any results obtained from the studies outlined in A and B. The basic technique for determining the copiness per cell of a particular virus nucleic acid is well worked out and has been used to determine the copiness of a variety of viruses in normal and transformed cells. The basis for this type of study involves measuring the kinetics of the formation of double stranded DNA molecules (or reassociation kinetics). The DNA of all mammals is composed of two fractions. One fraction is composed of DNA sequences which are present about 10^5 times per cell and is called the repeated DNA. The second fraction is composed of non-repeated DNA sequences which are present one time per haploid cell. This non-repeated DNA can be used as a calibration standard to determine the copiness of a particular sequence. The time necessary for a DNA sequence to reassociate is directly proportional to the concentration of the DNA sequences present in the reaction mixture. Mammalian DNA at a concentration of 10 mg/ml will reassociate in 1/10 the time that is needed by the same DNA at a concentration of 1 mg/ml. Thus, if a virus sequence is present in a cell at a concentration of 10 copies per haploid cell, it will take 1/10 less time for it to reassociate than it will for non-repeated DNA which is present at a concentration of 1 copy per haploid cell. If, however, a virus sequence is present at the same concentration as the non-repeated DNA sequences, one copy per haploid cell, it will take

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the same time to reassociate as does the non-repeated DNA. Thus, if one measures the time of reassociation of both the non-repeated DNA and the viral sequences, the copiness of the viral sequences can be determined. Viral sequence reassociation can be monitored by utilization of the same radioactive probe described in A and B. The non-repeated DNA reassociation can be followed by using non-repeated DNA labeled with a different isotope. Non-repeated DNA is readily separated from the repeated DNA.

VII. Search for viruses in continuous lymphocyte lines established from patients with systemic lupus erythematosus

As mentioned above, the autoimmune disease of the NZB and (NZB x NZW)F_1 mice has been the subject of intensive investigation because it is thought to be a very representative model of human systemic lupus erythematosus. In fact, many of the antibodies, which we have suggested are "immunologic tracks" and thus indicate that the host is defending against an RNA tumor virus, are present in these patients. Because of our successful attempt to demonstrate that a virus is present in every continuous lymphocyte line established from the New Zealand mice, a similar study will be carried out in patients with SLE. 25 patients with evidence of circulating antibody against SS DNA or dS DNA will be studied. The lymphocytes from 8 such patients are already in culture. Once established, the lymphoid lines will be studied for the presence of a virus utilizing methods already established for demonstration of the presence of virus in the murine system.

In addition, cell lines will be examined for the presence of complexes of 70S RNA and reverse transcriptase. This latter approach has been reported to be successful for demonstrating a 70S viral RNA associated with a reverse transcriptase in 95% of patients with acute or chronic myelogenous or lymphoid leukemia. If these studies are successful it may be possible to isolate a human tumor virus which would have obvious theoretical, diagnostic, and therapeutic implications.

VIII. Characterization of nucleic acid intermediates of RNA tumor viruses

The fact that antibodies against nucleic acids can be used to detect the presence of nucleic acid intermediates involved in the maturation of RNA tumor viruses opens the possibility that these antibodies could be used to isolate and quantitate the various nucleic acid intermediates involved in the replication of RNA tumor viruses (Tan and Lerner, J. Mol. Biol., 68:107, 1972).

To accomplish this, we plan to pulse label cells from continuously growing NZ murine lymphocyte lines with ^{3}H-thymidine and/or ^{14}C-uridine either in the presence or absence of 5 µg/ml of actinomycin D. Cells will be disrupted with .5% NP40 and the nuclei removed. This concentration of NP40 does not inhibit antigen-antibody union. The cytoplasm will be incubated at 37° for 30 minutes with 5 µl of human antibody against SS DNA, dS DNA, DNA-RNA hybrids, nuclear RNA, DNA-histone complex

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and anti-pyrimidine dimer. The human 7S antibody which will now hopefully be complexed to nucleic acids will be precipitated by addition of an excess of rabbit anti human IgG. The immune precipitates will be solubilized by treatment with 1% SDS and mercaptoethanol and then extracted with phenol to remove the protein. The nature of the nucleic acids in the precipitate will be then characterized by isopycnic centrifugation in cesium sulfate. In short, this method allows a "1 step" isolation of the intermediates involved in the replication of C type RNA tumor viruses.

In the procedures outlined above, the isolation of any DNA intermediate necessitates the use of antibody which recognizes a particular nucleotide sequence or conformation of the molecule and thus it is possible that certain molecules which any particular antibody does not recognize could be missed. Thus, we have developed a method to "photomark" DNA by induction of pyrimidine dimers. In this way antibody to pyrimidine dimers can be utilized as a generalized reagent to isolate any DNA containing intermediates. Recently other investigators have also demonstrated by fluorescent microscopy the specificity of anti-pyrimidine dimer antibody for DNA in cells which have been exposed to UV irradiation. In agreement with our results this reagent does not react with the DNA of cells which have not been exposed to UV irradiation.

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1. DNA is composed of 4 chemical subunits or nucleotides. A, G, C, T

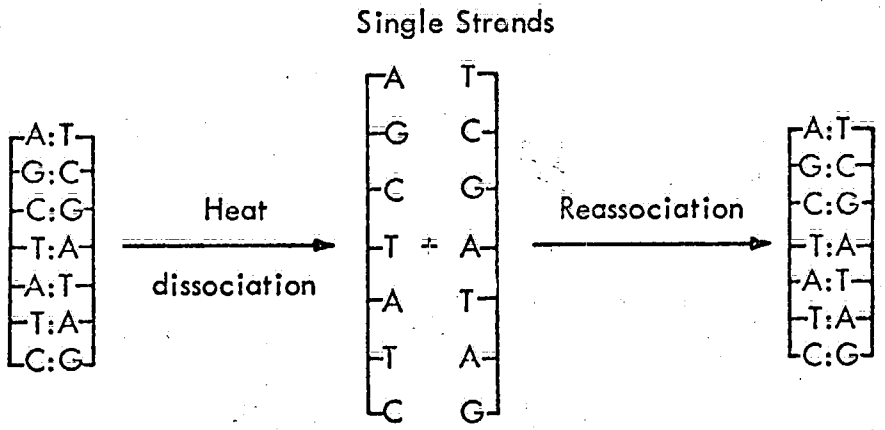
   Long linear polymers of these nucleotides constitute the DNA molecule which in its natural state is in a double strand form.

   The two strands are held together by specific interaction between the nucleotides in the two strands

   A interacts only with T
   G interacts only with C

2. In native DNA each nucleotide is paired with its complementary nucleotide in the other strand. The two strands are called complementary strands.

3. Individual strands can be separated by heating. Complementary single strands can reassociate to reform a double strand DNA. A DNA strand can recognize its complementary DNA strand and react with it.



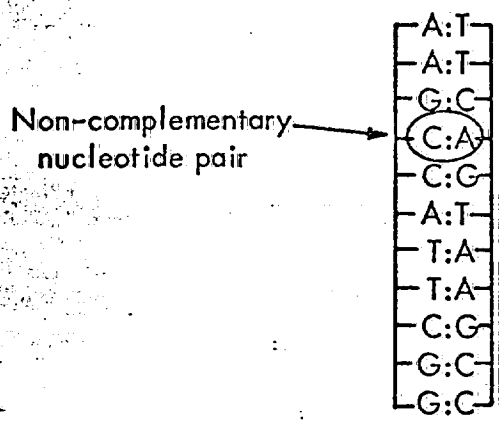
4. The reassociation reaction is a collision dependent reaction. The rate at which double strand molecules reform is then dependent on the concentration of each complementary strand in the solution.

FIGURE 1

Source: https://www.industrydocuments.ucsf.edu/docs/myyl0000

THERMAL STABILITY OF DNA

(1) Strands held together by specific hydrogen bonding between the bases in each strand. The double strand DNA is most thermal stable when all of the nucleotides are properly paired with their complementary nucleotide.



(2) When all of the nucleotides in the double strand region are not paired with their complementary nucleotide the thermal stability is lowered.

1.5% non-complementary nucleotide pairs	=	1°C lowering of thermal stability

(3) Two single DNA strands can be partially complementary and still reassociate to form a double strand molecule.

FIGURE 2

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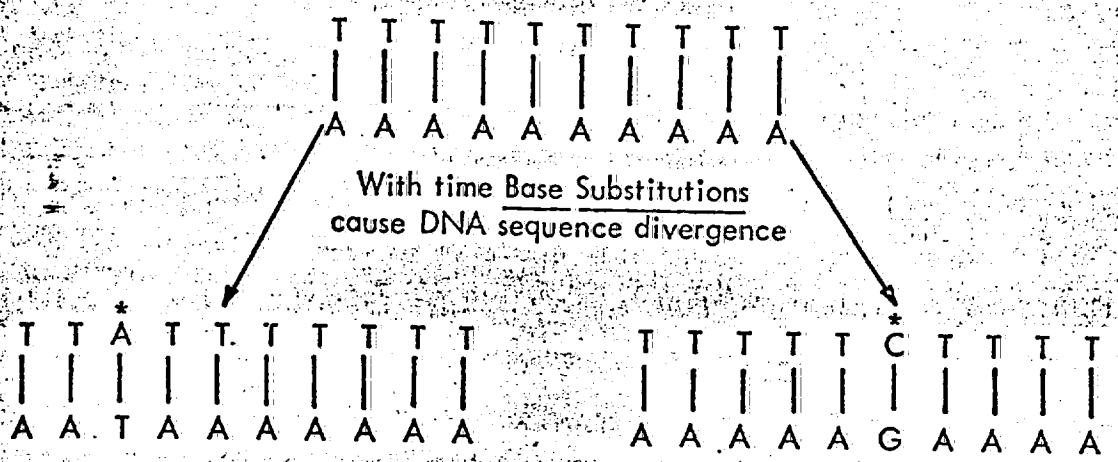
Source: https://www.industrydocuments.ucsf.edu/docs/myyl0000

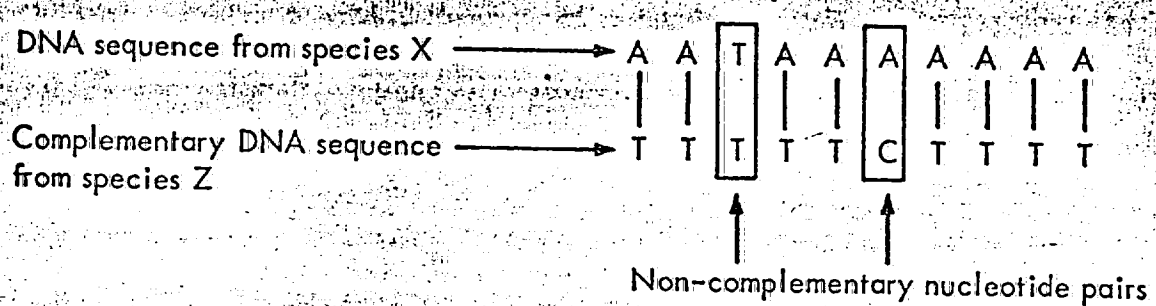
I. Original DNA sequence in the most recent common ancester of species X and Z.



Present day sequence in species X

Present day sequence in species Z

II. The DNA SEQUENCE from species X and Z can be compared in a test tube by forming a HYBRID DOUBLE STRAND DNA molecule. Single-strand DNA sequences do not have to be perfectly complementary in order to reassociate to form a stable double-strand hybrid molecule.



III. Double-strand DNA is most Thermal Stable when all of the nucleotides in one strand are properly paired with their complementary nucleotide.

When all of the nucleotides in the double-strand region are not paired with their complementary nucleotide, the thermal stability is lowered.

1.5% non-complementary nucleotide pairs = 1°C lowering of thermal stability.

IV. The percent of non-complementary nucleotide pairs seen for a particular hybrid represents the extent of nucleotide sequence change which has occurred since the time of divergence of the species involved.

*Nucleotide substitution

FIGURE 3

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Source: https://www.industrydocuments.ucsf.edu/docs/jkyb0000

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Source: https://www.industrydocuments.ucsf.edu/docs/myvl0000